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Evaluation of the drug synergistic and antibacterial effects of methanol extracts of *Callistemon viminalis* on some urinary tract infection bacteria

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ABSTRACT

Background and aims: Urinary tract infection is considered as one of the most common clinical problems in the world. The aim of this study was to investigate the interaction effects of pharmaceutical and antibacterial activity of methanol extracts of *Callistemon viminalis* on a number of bacteria that cause urinary tract infections.

Methods: In this experimental research, after drying leaves, extracts were prepared using methanol. The minimum lethal activity of 500, 250 and 125 µg/ml concentrations of the extracts on the standard bacteria was tested using disk diffusion and dilution method. In order to study the synergistic and antagonistic effect, a standard strains of bacteria was cultured on the medium containing methanol extract and then antibiotic discs were put on it.

Results: According to the results of disk diffusion test in Agar, the highest inhibition zone diameter in 500 µg/ml was related to *Staphylococcus epidermidis* and the minimum diameter in this concentration was related to *E. coli*. Study MIC results was showed that the extract of *Callistemon viminalis* has the highest inhibitory effect on *Staphylococcus saprophyticus* and *epidermidis*. The extracts of *Callistemon viminalis* showed synergistic effects with vancomycine and ciprofloxacin on *Staphylococcus aureus* and *epidermidis*, *Proteus vulgaris* and *Klebsiella pneumoniae*.

Conclusion: The results of this study indicate that extracts of *Callistemon viminalis* , alone or in combination with antibacterial agents may be useful in treatment of urinary tract infections. Additionally, this component can enhance the effect of some antibiotics; this implies its application, especially in drug resistance cases.

Keywords: *Callistemon viminalis* , Methanol extract, Antibacterial, Urinary tract infection, Disk diffusion.

Original article

INTRODUCTION

Plant derived products have been used for medicinal purposes for centuries. At present, it is estimated that about 80% of the world population rely on botanical preparations as medicines to meet their health needs.[1] The acceptance of traditional

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medicine as an alternative tretment and the development of microbial resistance to the classical antibiotics have led researchers to investigate the antimicrobial activity of several medicinal plants. Therefore, many reports of antimicrobial activity of plant extracts have been published in many regions in the world.[2,3] Today, despite the fact that a significant proportion of drugs are chemical, it is estimated that at least 30% of all pharmaceutical products either have a plant origin or after extraction from the plant, they are put in pharmaceutical formulations.[4] The genus *Callistemon* (Myrtaceae family) consists of 34 species native to Australia.[5] *Callistemon viminalis* species is commonly known as bottlebrush, It has 5 to 7 m height, producing bright red flower cluster with very rich in nectar. It is native to Australia, but now it is widespread throughout the world.[6,7] The bottlebrush plant resistance against drought, heat, wind and above all its beauty in time of budding, makes its wide usage in landscaping and roadside.[8] This species, *Callistemon viminalis* as a traditional medicinal herb, has a rich history in treating various diseases. Jamaica has a rich history in the use of diverse medicinal flora for traditional healing. These medicinal plants are generally used to treat various medical conditions including skin infections, stomach aches and respiratory conditions. It has been used to prepare a hot drink locally referred to as "tea" for the treatment of gastro-enteritis, diarrhea and skin infections.[9,10] *Callitemon veminalis* has a hemostatic property related to its astringent function that can halt the flow of internal bleeding, such as from ulcers, by constricting blood vessels. In addition its diuretic properties make it helpful in relieving water retention and general problems of the urinary tract. It is often used by women as a douche to clean the genito-urinary tract from excessive menstruation or a mucosal discharge known as leucorrhea.[11]

Urinary tract infections are common among the elderly, infants and especially women and it is one of the acute problems of organizations that are in charge of health in various countries.[12] Global statistics indicate that more than 250 million people are diagnosed with the disease in a year.[13] Common bacteria involved in this disease are *E. coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, and *Enterobacter*.[14]

Urinary tract infections are usually treated with antibiotics, but the report of pathogenic bacteria resistance to antibiotics is increasing.[15] So, new antibacterial and safe materials is more needed than before. Thus, the antimicrobial effects of natural herbs can pave the way for new antibiotics.[16] The use of medicinal plants by itself or in combination with modern medicine to reduce the side effects of the drug has been welcomed, The use of antibiotics in combination with plant extracts can help to prevent or delay the development of bacterial resistance to antibiotics.[17,18]

For this reason, we decided to study on the effects of anti-bacterial features of plant *Callistemon viminalis* on the common standard number of bacteria in the urinary tract infection. Then, we investigated the combined effects of methanol extract of *Callistemon viminalis* plant with two antibiotics, vancomycin and ciprofloxacin, to show this extract has a strengthening or an inhibitory effect on the function of the antibiotics. At last, the results of the study show this product is effective in combination with antibiotics and leads to treatment of urinary tract infections.

METHODS

In this experimental research, to study the antibacterial properties of the extract of

Callistemon viminalis, the standard strains of these bacteria, which are common in urinary tract infections, have been prepared in a lyophilized way in the microbiology laboratory of Fasa University of Medical Sciences: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 29212, *S.saprophyticus* ATCC 15305, *Enterococcus faecalis* ATCC 29212, *Proteus vulgaris* PTCC 1079, *Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 25922.

The leaves of *C. viminalis* were collected from the decorated trees in the city of Fasa in April 2015. The plant identity was confirmed in Department of Botany, Darab Payame Noor University, Iran. After cleaning, leaves were completely dried in the lab environment (away from direct sunlight and moisture) and then crushed to produce powdered material. Maceration method was used for the extraction. In fact, 20 grams of powdered leaves was soaked for 48 hours in methanol 80% (Merck of Germany) in order to have a maximum dissolution of active ingredients in methanol.

The extract was filtered in a Watman filter paper and then evaporated into a concentrated substance. Filtrates were then evaporated under reduced pressure and dried using a rotary evaporator at 55 °C. For a complete removal of the solvent, the samples were analyzed in 40 to 60 °C temperature in an oven. The plant extracts were prepared to be concentrated in 500 µg/ml and 250 µg/ml and 125 µg/ml. This means that a certain amount of the extract was weighed with a sensitive scale and dissolved in one milliliter of solvent, dimethyl sulfoxide 10% (Merck of Germany).[19]

Agar diffusion method is the most common method for evaluation of antimicrobial substances and known as Bayer-Krby test (Kirby Bauer). The base of this method is transfer of anti-bacterial material into the disc. In this method, at first, sterile blank disc (manufactured by Patton medicine) was placed in a solution of extract and after soaking, the discs were picked out of the solution and dried in an oven. The bacterial suspensions were prepared of bacterial strains and they were equivalent to 0.5 McFarland (10^8×1.5 CFU/ml). Then, a uniform cultivation was done by sterile cotton swabs soaked in suspension on the surface of Mueller-Hinton agar (Merck, Germany). Then, the disc containing the extract with concentrations of 500 µg/ml, 250 µg/ml and 125 µg/ml, was removed with sterile forceps and placed at a certain distance from each other, from the edge of the plates, on the surface of Mueller. The plates in an upside down state were incubated at 37 °C for 24 hours, and the diameter of inhibition zone around the disc were measured and sensitivity and resistance of strains were determined and then the results were compared with CLSI standard table.[20] In each experiment, a disc containing the solvent dimethyl sulfoxide (DMSO) 10% was determined as a negative control and a disc containing Vancomaycin 30 µg/disc and Ciprofloxacin 5 µg/disc was used as a standard antibiotic and positive control. The tests were repeated three times and the results were presented in the average of them.

MIC method extracts with dilution of the extract (250, 125, 62.5, 31.25, 15.6, 7.8, 3.9) were prepared. One ml of the diluted bacterial suspension equivalent to 0.5 McFarland (10^8×1.5 CFU/ml) is added to each dilution. Then all the test tubes are incubated at 37 °C, for 24 hours. After a period of time, Mueller-Hinton agar plates (Merck, Germany) were prepared, as the number of the tubes, and cultured was uniform.[21] The lowest concentration of the extract which inhibits the growth of bacteria was reported as the MIC.

To determine the combined effect between methanol extract of bottlebrush and antibiotic disks, was used disc diffusion

method. The examination of synergistic and decreasing effects of extracts on antibiotic is done under inhibitory concentrations (sub-MIC). Concentration of the extracts under inhibitory state to the Mueller-Hinton agar is added with a ratio of 1 to 2 (1 unit extract and 2 units Mueller-Hinton Agar medium) were used as plate test. Bacterial suspension, with a concentration of 0.5 McFarland (10^8×1.5 CFU/ml), is cultured on Mueller-Hinton Agar medium containing inhibitory concentrations of the extract in a grass-like state. Antibiotic discs were placed on the surface of the medium. The disc containing solvent is determined as negative control and a disc with extract and an inhibitory concentration (sub-Mic) was recorded as positive control. Then the diameter of zone growth inhibition was measured after 24 hours of incubation at 37 °C. To study on the interactions effect between of methanol extract of bottlebrush and antibiotic, was used Vancomaycin 30 µg/disc and Ciprofloxacin 5 µg/disc antibiotic disc.[22]

Statistical Analysis: experiments were based on a repeated triple testing. The results were averaged and standard deviation of the changes was estimated. A comparison of the antibacterial activity of the samples with standard antibiotics was evaluated by applying t-test and ANOVA (software SPSS). All values are expressed as the mean and $P<0.05$ values were considered to indicate statistically significant differences.

RESULTS

The impact of two common antibiotics and 3 *Calistemon viminalis* concentrations on the inhibition 4 Gram-positive and 3 Gram-negative is presented in Table1 and show that all bacteria were susceptible in tested concentrations. The bacteria type and concentration of the extract were effective on bacteria inhibition zone diameter. In other words, the highest inhibitory effect is seen in gram-positive bacteria and with increased concentration, its antibacterial activity enhanced against tested bacteria. According to the test disc diffusion, the most diameter of the inhibitory zone between Gram positive and negative at concentrations 500 µg/disc were observed in *S.epidermidis* with a diameter of 22 mm and then the maximum diameter of the growth of *Staphylococcus saprophyticus*, *Enterococcus faecalis* and *Klebsiella pneumoniae*, and *E. coli* have the lowest inhibitory zone diameter of 7.8 mm (Tabel 1). The Study results show that MIC *Calistemon viminalis* highest inhibitory effect is seen among *Staphylococcus epidermidis* and *saprophyticus* (Table 2).

Table 1: Agar disk diffusion test in standard bacterial strains for the extract *Calistemon viminalis*

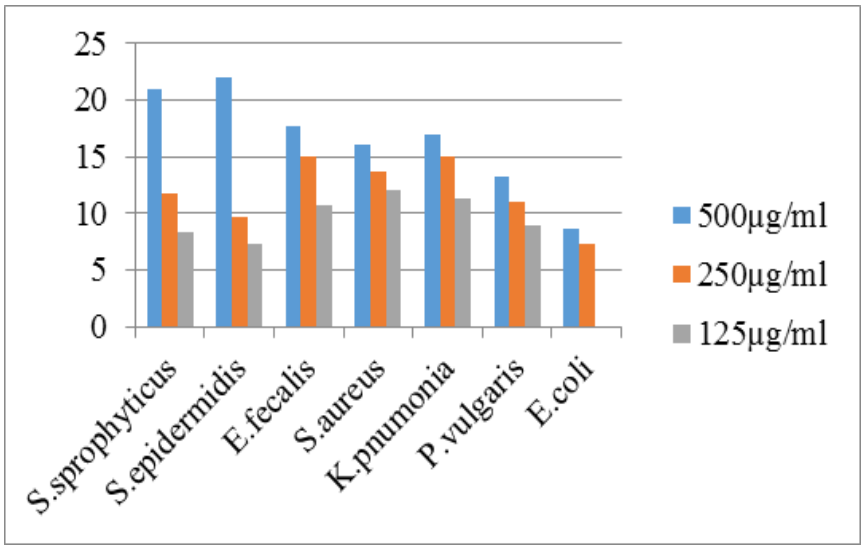
Bacteria	**Agar disk diffusion (mm)**					
	Extract *Callistemon viminalis*			Positive control		Negative control
	500 µg/disc	250 µg/disc	125 µg/disc	Ciprofloxacin	Vancomycin	
S. saprophyticus	21 mm	19 mm	15.3 mm	14 mm	23 mm	-
S. epidermidis	22 mm	16.3 mm	13 mm	12 mm	24 mm	-
E. faecalis	17.7 mm	15 mm	10.7 mm	16 mm	21 mm	-
S. aureus	16 mm	13.7 mm	12 mm	12 mm	22 mm	-
K. pneumoniae	17 mm	15 mm	11.3 mm	-	29 mm	-
P.vulgaris	13.3 mm	11 mm	9 mm	-	25 mm	-
E. coli	8.7 mm	7.3 mm	-	-	24 mm	-

(-): Not growth bacteria.

Table 2: Minimum Inhibitory Concentration (MIC) of methanol extracts of plants *C. viminalis* on standard bacterial strains

Concentration / Bacteria	Concentration extract µg/ml						
	250	125	62.5	31.25	15.6	7.8	3.9
S. saprophyticus	-	-	-	-	-	+	+
S. epidermidis	-	-	-	-	-	+	+
E. faecalis	-	-	-	-	+	+	+
S. aureus	-	-	-	-	+	+	+
K. pneumoniae	-	-	-	-	+	+	+
P.vulgaris	-	-	+	+	+	+	+
E. coli	-	+	+	+	+	+	+

(-): Not growth bacteria; (+): Growth bacteria.



Graph 1: Compares the antibacterial effect of the extracts

As Graph 1 shows, there is an association between inhibition zone and extract concentration. In other words as the concentration increased, the antibacterial effect increased too.

The statistical results of the T-test show that the antibacterial effects of extracts in meaningful level of 0.02 ($P<0.05$) are related to concentration dependent, and always with the increase of the concentration, the diameter of the inhibitory zone is also increased. This can be attributed to the amount of active ingredient in the extracts and this amount is more in methanol extracts. It also shows the significant difference at level 5% in the inhibition growth diameter among all bacteria in the presence of extracts and control.

The results of the effects of synergistic and antagonistic of the extract on two antibiotics for 7 strains of bacteria using standard is given in Table 3. According to the diameter of growth inhibition, it is observed that the only antagonistic effect of

the extract is seen in conjunction with vancomycin on *Staphylococcus saprophyticus* and also in *E. coli* in interaction with ciprofloxacin.

Table 3: Comparison of the effects of *C. viminalis* extract with antibiotics

Bacteria	*S. aureu*	*S.epidermidis*	*E. faecalis*	*S. saprophyticus*	*P.vulgaris*	*K. pnumonia*	*E. coli*
Vancomycin	S	S	S	A	S	S	I
Ciprofloxacin	S	S	S	I	S	S	A

S: Synergistic effect; A: Antagonistic effect; I: intermediate (ineffective).

DISCUSSION

The results showed that the plant *Calistemon viminalis* has an antimicrobial activity and among the tested bacteria, in comparison with Gram-positive bacteria such as *Staphylococcus saprophyticus* and *epidermidis*, and in gram-negative bacteria such as *E. coli* is more vulnerable to extract inhibitory property. This can be related to the lipopolysaccharide external membrane of Gram-negative bacteria that make them inherently resistant to external factors such as hydrophilic dyes, antibiotics and detergents.[23]

The presence of antimicrobial compounds in plants represents them as a useful case for the development of natural products and an alternative to antibiotics. In addition, they provide a basis for the development of new antibiotics.[24]

The antimicrobial feature of plants is generally due to the presence of phenolic compounds, saponins, flavonoids in their structure and some of these factors can affect on plasma membrane or the containment structure enzymes of cell membranes of microorganisms and can apply their antimicrobial properties, The previous phytochemical researches led to the identification of C-methyl flavonoids, terpenoids and derivatives of phloroglucinol.[25-29] Terpenoids compounds in *Callistemon viminalis* have a sharp taste and in this case, they have been detected as anti-microbial.[30]

The antimicrobial activity of essential oils of *C. viminalis* can be due to presence of some major compounds such as 1,8- Cineol, α-pinene, α-terpineol along with smaller amounts of other compounds such as β-pinene and linalool that their antimicrobial activity and bacteriostatic state has been detected.[31-34]

In 2009, Delahaye and colleagues found that compounds in the plant *C. viminalis* has an antibacterial activity against *Staphylococcus aureus* and *E. coli* with an inhibitory zone diameter about 16-20 ml, the results of this study about *Staphylococcus aureus* match with the results of the study in 2009.[35] But in the case of *E. coli*, the obtained growth inhibitory zone diameter in this study is less than that study, and also, according to Oyedeji and colleagues research in 2009, it is showed that the bottlebrush extract has antibacterial properties against a number of bacteria such as *E. coli, S. aureus, K. pnumonia, P.vulgaris*.[36] The findings of the present study correspond with the results of Oyedeji. Shinde and colleagues in 2012, determined that the bottlebrush has antibacterial properties against bacteria *E. coli, B.subtilis* and *P.aeruginosa* and it showed that the plant is a medicinal plant with medical

applications. that test results correspond with the present study findings.[37] In 2010, Quijano-celis and colleagues determined that the plant has an antibacterial activity against *Staphylococcus aureus* with a diameter greater than 20 mm and the test results are compatible with the results of the present study.[38]

CONCLUSION

The result of this study indicates that extracts of *callistemon viminalis*, alone or in combination with antibacterial agents may be useful in treatment of urinary tract infections. Additionally, this component can enhance the effect of some antibiotics; this implies its application, especially in drug resistance cases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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